Kidney International, Vol. 34 (1988), pp. 117–120

TECHNICAL NOTE

Measurement of urinary para-aminohippuric acid in glycosuric diabetics

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The observation that hyperglycemia may reduce the renal clearance of para-aminohippuric acid (PAH) dates back to 1944 [1]. This and similar findings [2, 3] have been interpreted as the result of physiological competition between renal tubular glucose reabsorption and PAH secretion [4]. Similar problems have, however, not been encountered when the clearance of radioactive iodohippuran has been used to estimate renal plasma flow (RPF). The different results obtained using different techniques have contributed to the considerable uncertainty surrounding the evaluation of RPF in diabetics who have been reported as either having normal [5], high [6] or low [7] RPF. Recently, Dalton et al [8], in a study of renal hemodynamics in normal subjects at different levels of stable hyperglycemia, found that a marked fall in the clearance of free PAH occurred only in the presence of glycosuria. In a preliminary study in our laboratory we observed a physiologically improbable fall in PAH clearance during hyperglycemia and glycosuria in insulin-dependent diabetics. Urine aliquots taken into tubes containing sodium hydroxide (NaOH) to measure urinary concentrations of β_2-microglobulin, a protein which is unstable at low pH, yielded PAH concentrations in the expected range.

To clarify the reasons for these findings we have performed a series of in vivo and in vitro studies which were aimed at explaining the fall of free PAH concentrations in urine during hyperglycemia with glycosuria, and at exploring possible solutions to the fluctuation.

Methods and patients

Six male insulin-dependent diabetics performed the study which was approved by the Guy's Hospital Ethical Committee. They had no evidence of clinical proteinuria, were aged between 18 and 33 years (mean 26 years), and with duration of diabetes ranging between 6 and 14 years (mean 9 years).

Patients were maintained euglycemic by an insulin infusion overnight, and rendered hyperglycemic by a graded intravenous dextrose infusion the morning of the test [9]. A classical constant infusion renal clearance procedure was then performed during a steady state of water diuresis, using PAH (Sodium Aminohippurate, Merck, Sharp & Dohme Ltd, Hoddesdon, UK). After 45 minutes equilibration, urine was collected during six consecutive, accurately timed clearance periods of approximately 20 minutes each and blood was taken at the mid-point of each clearance period for measurement of PAH and glucose. Two 3.5 ml aliquots of urine were taken; one without additives, and one collected into a tube containing 20 μl of 4 M NaOH. The pH of this latter collection exceeded 9 in the majority of the samples. The samples were then frozen within 6 hours and kept at −20°C until assayed, between 8 and 26 weeks later.

Prior to PAH assay 200 μl of each untreated urine sample were mixed with 10 μl of 0.4 M NaOH or 10 μl concentrated (11.6 M) hydrochloric acid (HCl, Analar) in capped polypropylene tubes and heated in a dry block for 10 minutes at 70°C. The samples were then cooled and mixed. Thus, PAH was measured in untreated urine samples, in urine collected in NaOH and in urine treated after storage with NaOH or HCl.

Addition of PAH and glucose to control urine

A solution containing 58.2 mg/dl PAH and 360 mg/dl glucose was made up in normal control urine diluted 1:10 in distilled water (final creatinine concentration 0.9 mmol/liter), to simulate the sample conditions associated with forced diuresis. The pH of this solution was 5.9. Several 3.5 ml aliquots of this solution were prepared and 20 μl of 4 M NaOH was added to half of them. The pH of aliquots containing NaOH was greater than 11. PAH was measured after incubation at 37°C for 20 minutes (to simulate the in vivo period during which urine resides in the bladder at body temperature) in untreated urine and then after six hours at room temperature and 1, 3, 8, and 26 weeks at −20°C in both untreated urine and urine containing NaOH. As control experiments aliquots of urine from control and glycosuric diabetic subjects not receiving PAH infusion as well as aliquots of an aqueous solution (pH 3.1) containing PAH (100 mg/dl) and glucose (1.8 g/dl) were stored at −20°C for 20 weeks and assayed by high pressure liquid chromatography (HPLC). All determinations were made in triplicate.

Measurements

PAH was measured according to the method of Brun [10], adapted for use on a centrifugal analyzer (COBAS Bio, Roche Diagnostica Ltd, Welwyn Garden City, UK) and directly on centrifuged urine samples by HPLC [11]. The detection wavelength was 275 nm and the acetonitrile concentration in the elution buffer was increased to 5.7% for optimal separation of free PAH and PAH-glucose conjugate (PAH-G).

Received for publication September 18, 1987
and in revised form February 28, 1988

Table 1. Plasma and urine concentrations of glucose and PAH in 6 insulin-dependent diabetic patients

Subject	Glucose *mmol/liter*		PAH *mg/dl*					Storage time *weeks*
	Plasma	Urine	Plasma	Urine collected in NaOH	Urine untreated	Urine treated with NaOH	Urine treated with HCl	
1	15.1	15.6	1.3	67.1	4.8	4.8	68.6	26
2	15.0	22.7	1.6	109.3	11.2	10.8	107.3	22
3	13.6	27.9	1.5	112.1	28.4	21.2	117.4	8
4	13.0	28.2	2.3	89.5	8.9	7.4	87.0	14
5	16.7	19.5	1.3	81.9	11.1	9.6	87.2	21
6	15.1	23.4	1.5	83.6	13.7	13.9	87.4	17
Mean	14.8	22.9	1.6	90.6	13.0	11.3	92.5	
SEM	0.5	2.0	0.2	7.0	3.3	2.3	7.1	

Urines were collected in NaOH, untreated, and treated with NaOH or HCl before assay.

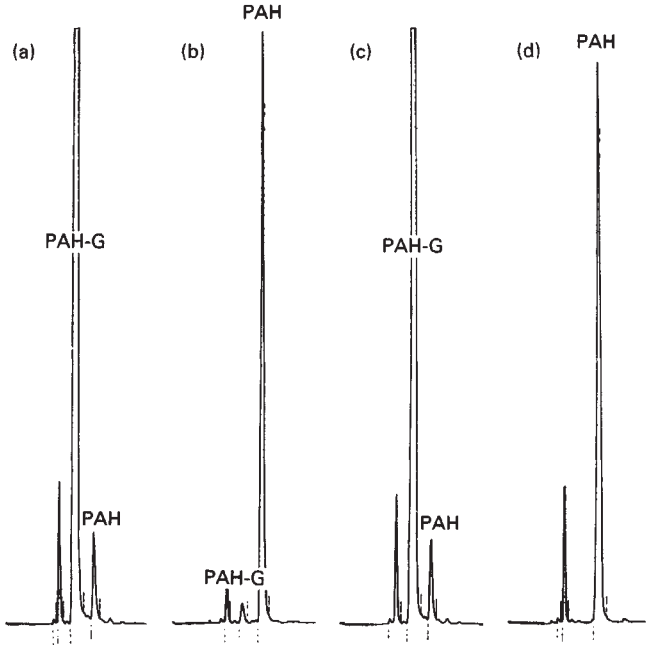

Fig. 1. *HPLC traces of PAH and PAH-glucose (PAH-G) conjugate in* **(A)** *untreated urine,* **(B)** *urine collected in NaOH,* **(C)** *urine treated with NaOH after storage, and* **(D)** *urine acid hydrolyzed after storage.*

Glucose was measured in plasma and urine by a glucose oxidase method (Yellow Springs YSI 23AM, Yellow Springs, Ohio, USA). In the studies in diabetic patients the mean urine and plasma concentrations of PAH and glucose of all six collection periods for each patient are presented. Data were analyzed using the paired *t*-test and the significance level taken at 1% because of multiple comparisons.

Results

Table 1 shows plasma and urinary glucose concentrations and the levels of PAH in plasma and in urine collected in NaOH, untreated, and treated with NaOH or HCl before assaying.

All six subjects were hyperglycaemic (range 13.0 to 16.7 mmol/liter) and had significant glycosuria (range 15.6 to 28.2 mmol/liter). Mean plasma PAH was 1.6 mg/dl (range 1.3 to 2.3). In the urine, PAH concentrations were significantly lower ($P < 0.001$) in the untreated aliquots compared to those collected into NaOH. Addition of NaOH to the untreated urine before assay did not result in recovery of PAH. By contrast, acid hydrolysis of the untreated urine prior to assay led to PAH concentrations strictly comparable to those found in urine collected in NaOH.

Table 2. The effect of storage with and without NaOH on measurable free PAH in a urine solution containing PAH (58.2 mg/dl) and glucose (360 mg/dl)

Time and conditions of storage	PAH *mg/dl*	
	Urine untreated	Urine with NaOH added
20 min at 37°C	59.7	—
6 hrs at room temperature	60.4	59.6
1 week at −20°C	53.5	60.4
3 weeks at −20°C	59.4	59.8
8 weeks at −20°C	35.8	62.1
26 weeks at −20°C	17.5[a]	59.7

[a] This sample was hydrolyzed with HCl as described in methods and reanalyzed PAH was 58.8 mg/dl.

Figure 1 shows the results obtained when urinary PAH was determined by HPLC. In all the untreated urine samples free PAH was extremely low, confirming the results obtained using the Brun method. In these urines a second peak (PAH-G), chromatographing slightly earlier than PAH, was observed. This peak was virtually absent in the urine samples collected in NaOH or those subsequently acid hydrolyzed, but it was present in the urine samples treated with NaOH prior to assay. The elution fraction corresponding to the PAH-G peak was collected and analyzed for PAH and glucose; both were barely detectable. Following acid hydrolysis of the peak fraction equimolar amounts of glucose and PAH were recovered.

Table 2 shows the effect of storage with and without NaOH of a control urine solution containing PAH (58.2 mg/dl) and glucose (360 mg/dl). After 8 and 26 weeks of storage at −20°C, measured free PAH concentrations in the urine without NaOH were 40% and 70% lower, respectively, than the initial PAH value. By contrast, no change in PAH concentration was detected in the urine solution containing NaOH. At 26 weeks the untreated urine was acid hydrolyzed, which led to total recovery (58.8 mg/dl) of the free PAH. HPLC analysis of these samples confirmed the loss of free PAH in the untreated urine and the appearance of the PAH-G peak. No peak with this retention time was seen in the chromatograms of stored urine from control or glycosuric diabetic subjects not containing PAH, but a similar peak was present in the aqueous solution of PAH and glucose stored at −20°C (data not shown). Acid

hydrolysis resulted in the loss of the PAH-G peak and recovery of free PAH and glucose confirming that the PAH-G peak represents an adduct of PAH and glucose.

Discussion

This study demonstrates that the lower free PAH concentrations found in glycosuric urine in man are the result of reaction between glucose and PAH during storage. That PAH and glucose might react, presumably to form a Schiff base, was originally suggested by Baldwin et al [12] who noticed a fall in PAH concentration in solutions of glucose and PAH prepared for infusion. Recently, Lote, McVicar and Yardley [13] confirmed that glucose and PAH will react in vitro, but considered the reaction too slow to be a problem under most experimental conditions. In their physiological studies in hyperglycemic rats they concluded that the fall in PAH clearance resulted from competition between renal tubular reabsorption of glucose and secretion of PAH, in agreement with previous views [4].

In man, however, a series of observations argue against this interpretation. Firstly, the fall in PAH clearance observed during hyperglycemia only occurs when the hyperglycemia is accompanied by glycosuria and is due to an apparent decrease in urinary PAH [8], an effect which could be abolished by acid hydrolysis [14]. No loss of urine free PAH was seen in either normoglycemic or hyperglycemic non-glycosuric subjects [8]. Secondly, no effect of hyperglycemia or glycosuria has been recognized when renal plasma flow is measured using radioactive iodohippuran [6], although PAH and iodohippuran are both secreted by the weak organic acid transporter. Finally, the renal extraction of PAH, measured using classical renal arteriovenous PAH concentration differences, has been shown to be normal in diabetes [15].

Our findings with HPLC analysis of urine PAH demonstrating the existence of a peak in glycosuric urine, which may be hydrolyzed to glucose and PAH, strongly suggest the formation of a PAH adduct, probably a Schiff base. The PAH and glucose are reacting with each other is further supported by our experiment showing the formation of the PAH-G peak in an aqueous solution containing PAH and glucose alone. Chemical methods for the measurement of PAH, such as those described by Bratton and Marshall [16] and Brun [10], depend on the availability of the para-amino group for reaction. Schiff base formation between the para-amino group of PAH and the aldehyde group of open chain glucose would occlude the amine group and reduce measurable free PAH.

Collection of urine in NaOH or acid hydrolysis of stored glycosuric urine maintained and respectively restored the expected concentrations of PAH. HPLC analysis of urine submitted to these procedures showed a virtual abolition of the PAH-G peak seen in untreated glycosuric urine. Schiff base formation is a reversible reaction dependent on initial nucleophilic attack by the free amine group of PAH on the aldehyde group of open-chain glucose. This step is promoted in alkaline conditions, whereas the final dehydration step is dependent on acid conditions. Alkalinization of urine to a pH of 9 or greater prevented the reaction totally, presumably by providing conditions in which the final dehydration step could not take place. Certainly, alkaline hydrolysis of the complex does not take place since alkalinization of the urine after the reaction between PAH and glucose has occurred is unable to reverse it. By contrast acid hydrolysis restores free PAH by reducing the availability of the nucleophile and allowing the back reaction to predominate.

The ability of NaOH to prevent the formation of a PAH-G peak excludes the possibility of pre-renal or intra-renal reaction between glucose and PAH as an explanation of our data. In this communication the various factors which influence the reaction between PAH and glucose, such as concentration, pH, temperature, and time, have not been considered in detail, but both the in vivo and in vitro studies demonstrate that measurable free PAH declines in glycosuric urine stored at −20°C. Under our experimental conditions several weeks were required to detect a fall in free PAH concentration. There was no detectable loss at 37°C or room temperature within the time restrictions of our in vitro experiment.

In conclusion, this series of experiments taken together support the notion that no significant impairment of tubular PAH secretion takes place during hyperglycemia. The loss of free PAH in glycosuric urine, and the resultant apparent lowering of PAH clearance, occurs in vitro during storage and not in vivo during the clearance procedure. The in vitro conversion of free PAH to an analytically silent adduct can be prevented by alkalinizing the urine immediately after collection. Once the reaction between PAH and glucose has taken place, recovery of free PAH can be achieved by acid hydrolysis of the urine prior to assay.

Acknowledgments

We thank Professors H. Keen and C. Chantler for their support, the staff of Peter Bishop Metabolic Ward, and our patients for their help. This study was supported in part by the Children Nationwide Medical Research Fund and the Wellcome Trust.

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References

1. Klopp C, Young NF, Taylor HC Jr: Probable errors in the simultaneous measurement of separate kidney functions. *J Clin Invest* 29:117–121, 1944
2. Houck CR: Mutual depression of reabsorption and excretory maxima in renal tubules. *Proc Soc Ex Biol Med* 63:398–401, 1946
3. Grimelli LJ, Chertack MM, Rhetta HL, Kendrick AB, Forrest RA: Effect of hyperglycaemia on the clearances of inulin and para-aminohippuric acid. (abstract) *J Lab Clin Med* 33:1617, 1948
4. Smith HW: *The Kidney: structure and function in health and disease*. New York, Oxford University Press, 1951, p. 202
5. Mogensen CE, Andersen MJF: Increased kidney size and glomerular filtration rate in early juvenile diabetes. *Diabetes* 22: 706–713, 1973
6. Christiansen JS, Gammelgaard J, Frandsen M, Parving H-H: Increased kidney size, glomerular filtration rate and renal plasma flow in short-term insulin-dependent diabetics. *Diabetologia* 20:451–456, 1981
7. Ditzel J, Junker K: Abnormal glomerular filtration rate, renal plasma flow, and renal protein excretion in recent and short-term diabetics. *Br Med J* 2:13–19, 1972
8. Dalton RN, Turner C, Greene S, Haycock GB, Chantler C: Schiff base formation between glucose and para-aminohippurate: A serious artefact in the measurement of the renal clearance of para-aminohippurate. (abstract) *Diabetic Med* 4:38, 1987
9. Wiseman MJ, Mangili R, Alberetto M, Keen H, Viberti GC: Glomerular response mechanisms to glycemic changes in insulin-

dependent diabetics. *Kidney Int* 31:1012–1018, 1987
10. BRUN C: A rapid method for the determination of para-aminohippuric acid in kidney function tests. *J Lab Clin Med* 37:955–958, 1951
11. PRUEKSARITANONT T, CHEN ML, CHIOU WL: Simple and micro high-performance liquid chromatographic method for the simultaneous determination of p-aminohippuric acid and iothalamate in biological fluids. *J Chromatogr* 306:89–97, 1984
12. BALDWIN DS, SCHREINER GE, BREED ES, WESSON LG JR, MAXWELL MH: Depression of apparent p-aminohippurate extraction ratio by glucose. *J Clin Invest* 29:614–618, 1950
13. LOTE CJ, MCVICAR AJ, YARDLEY CP: Renal extraction and clearance of p-aminohippurate during saline and dextrose infusion in the rat. *J Physiol* 363:303–313, 1985
14. GREENE SA, DALTON RN, TURNER C, HAYCOCK GB, CHANTLER C: Hyperglycemia with and without glycosuria: Effect on inulin and para-amino hippurate clearance. *Kidney Int* 32:896–899, 1987
15. NYBERG G, GRANERUS G, AURELL M: Renal extraction ratios for ^{51}Cr-EDTA, PAH, and glucose in early insulin-dependent diabetic patients. *Kidney Int* 21:706–708, 1982
16. BRATTON AC, MARSHALL EK JR: A new coupling component for sulfanilamide determination. *J Biol Chem* 128:537–550, 1938